

Effects of interactions of hepatocyte nuclear factor 4α isoforms with coactivators and corepressors are promoter-specific

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Abstract The gene encoding hepatocyte nuclear factor 4α (HNF4α) possesses two alternative promoters responsible for developmental and tissue-specific expression of HNF4α1 and HNF4α7. The two isoforms possess different N-termini and exhibit distinct transactivation properties. We show here for the first time that the effects mediated by HNF4α isoforms in concert with three different coregulators result in promoter-specific responses. Transcript levels of silencing mediator for retinoid and thyroid receptors and glucocorticoid receptor interacting protein-1 in the liver are reduced at birth, a time point when many genes are strongly activated, suggesting that the effects of coregulators on HNF4α activity in vivo could be determined by the levels of their expression as well as by the target promoter. © 2003 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Hepatocyte nuclear factor 4α; Hepatocyte nuclear factor 4α isoform; Apolipoprotein; Liver transcription; Glucocorticoid receptor interacting protein-1; Silencing mediator for retinoid and thyroid receptors; HNF4

1. Introduction

The gene encoding the nuclear receptor hepatocyte nuclear factor 4α (HNF4α) [1] possesses two alternative promoters [2], the use of which is responsible for developmental and tissue-specific expression of the HNF4α1 and HNF4α7 isoforms [3,4]. The latter isoform is expressed predominantly in the embryonic/fetal liver while only HNF4α1 is found in the adult organ. The two isoforms possess intrinsic differences in their transactivation properties [4]. Indeed, the hepatic functions expressed in the embryonic liver are mostly in common with those of the adult, but at birth, a whole new set of functions, implicated in adult hepatic metabolism, is activated. The fetal isoform transactivates preferentially promoters of

fetal functions while the adult isoform is more active on promoters of genes expressed more avidly in the adult [4].

It is known that HNF4α, like other nuclear receptors, provokes gene activation in concert with interactions with coactivators and corepressors through its activation function (AF) domains. The ability of HNF4α1 and HNF4α7 to interact with the CREB binding protein (CBP)/p300 and p160 coactivators and with silencing mediator for retinoid and thyroid receptors (SMRT) corepressor has been explored using mainly transactivation assays based on the Gal4 one-hybrid system [5–8]. HNF4α1, via both the AF1 and AF2, shows enhanced transactivation of reporter constructs in the presence of CBP or glucocorticoid receptor interacting protein-1 (GRIP-1), and exhibits, via the AF2, SMRT-mediated transrepression. HNF4α7 shows similar properties, but only via the AF2, the AF1 being absent from this isoform [7].

In this work, we explored the possible role that interactions with these coregulators play in determining fine tuning of HNF4α activity on different target promoters by cotransfection experiments using different reporters for HNF4α. We report that the activities elicited by these coregulators on HNF4α-dependent transcription are dependent on the target promoter. Interestingly, it was observed that patterns of transactivation/transrepression were dictated by the target promoter and in almost all cases the effects mediated by coregulators in concert with HNF4α1 were more robust than those observed with the same coregulators and HNF4α7.

2. Materials and methods

2.1. Plasmids

The empty pCB6 vector [9] and the CMV.HNF4α1.VSV [10] and CMV.HNF4α7.VSV vectors [4] containing the full-length rat cDNAs for the corresponding isoforms as well as the apolipoprotein (Apo) AI reporter pZL.HIV.LTR.AI-4 [11], the ApoCIII.Luc reporter [12] and the ApoB reporter [13] have all been described. Detailed description of these reporters is provided in the legend to Fig. 1. The pSG5.SMRT [14], pSG5.GRIP-1 [15] and pCMV.HA.p300 [16] expression vectors have been described elsewhere. The CMV.βGal plasmid (California Biotechnology) contains the *lacZ* gene under control of the cytomegalovirus (CMV) promoter.

2.2. Transient transfections

HNF4α-deficient 293T [17] cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum at 37°C under a 5% CO₂ atmosphere. For transient transfection assays, 2 × 10⁵ cells were seeded in six-well plates and were transfected by the calcium phosphate coprecipitation procedure 1 day later using 1 μg of reporter, 100 ng of CMV.βGal and various amounts of expression vectors as indicated in the figure legend. Equivalent molar amounts of empty vector were added to equalize DNA amounts. Glycerol shock was carried out 5 h later and cells were harvested 24 h after the shock.

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Abbreviations: HNF4, hepatocyte nuclear factor; AF, activation function; CBP, CREB binding protein; GRIP, glucocorticoid receptor interacting protein; SMRT, silencing mediator for retinoid and thyroid receptors; Apo, apolipoprotein; RT-PCR, reverse transcription-polymerase chain reaction

β -Galactosidase activity was measured by the standard colorimetric method and luciferase activity was determined with a Berthold 9501 luminometer as described [18].

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA extraction from mouse liver and RT-PCR were carried out as previously described in the presence of 1 μ Ci [α - 32 P]dCTP [4]. Primers used for SMRT cDNA amplification were 5'CACCCCA-

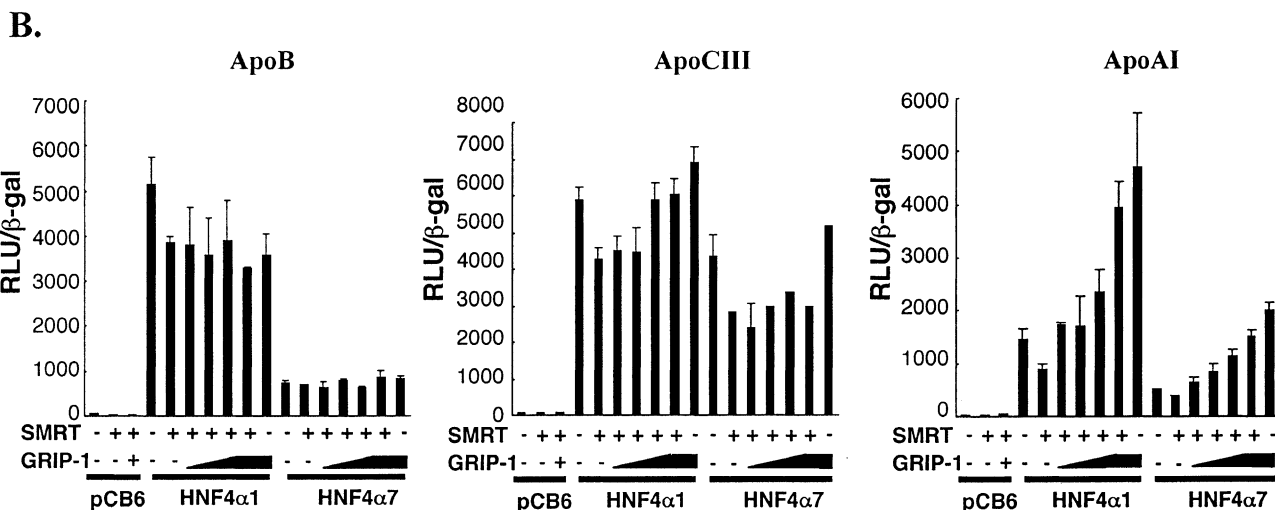
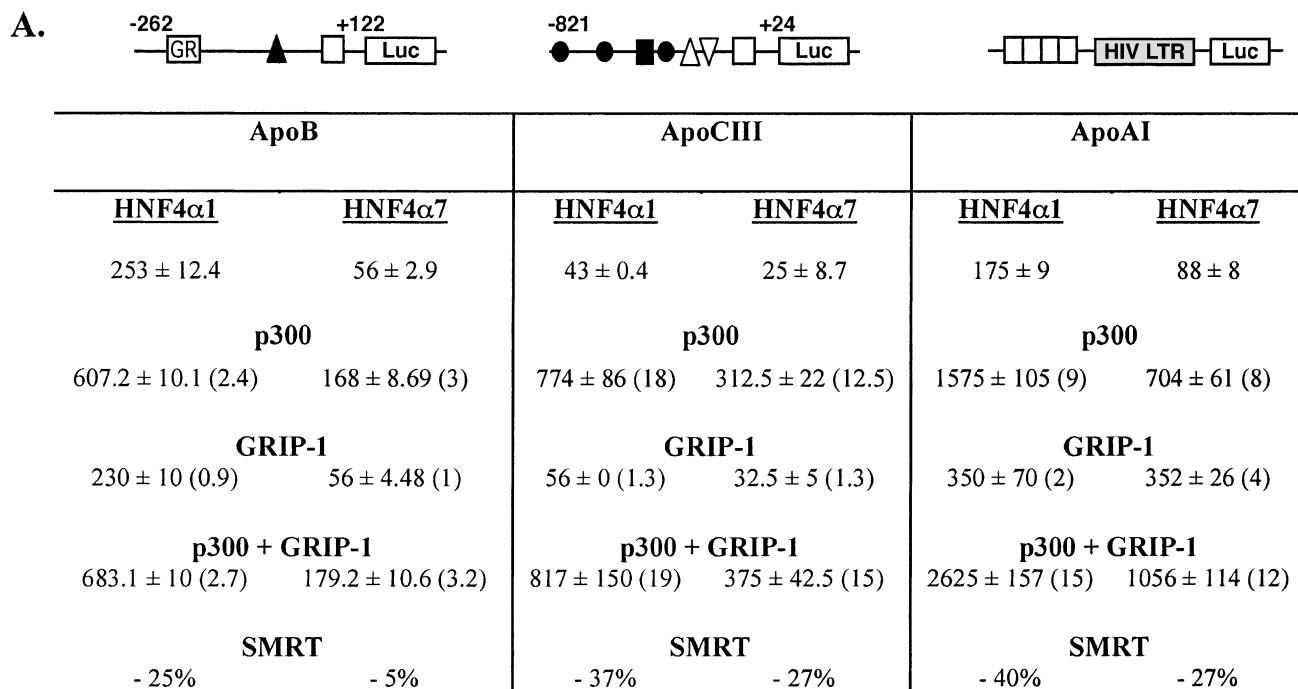


Fig. 1. A: Effects of p160 and p300 coactivators and SMRT corepressor are isoform- and promoter-specific. 293T cells were cotransfected with 20 ng of empty (pCB6), HNF4 α 1 or HNF4 α 7 expression vectors and the ApoB, the ApoCIII or the ApoAI reporter together with 100 ng of the CMV.p300.HA and/or the pSG5.GRIP-1 plasmid. For the transfections with SMRT, 50 ng of the HNF4 α expression vectors together with 250 ng of pSG5.SMRT were used. Schematic representation of each of the reporters used is shown. The ApoB reporter contains the region -262 to +122 from the human ApoB promoter linked to the firefly luciferase gene. The ApoCIII reporter contains the region -821 to +24 from the human ApoCIII gene driving the expression of the luciferase gene. The ApoAI reporter consists of four site A elements responsive to HNF4 α from the human ApoAI promoter in front of the human immunodeficiency virus long terminal repeat driving expression of the luciferase gene. Binding sites for other transcription factors are represented as follows: GR glucocorticoid receptor, \blacktriangle AP2, \blacksquare ARP1, \bullet Sp1, \triangle NF- κ B, ∇ C/EBP, \square HNF4. Luciferase activity was normalized to β -galactosidase activity. Numbers give the fold induction by HNF4 α 1 or HNF4 α 7 on reporter gene activity relative to that with the empty vector and the fold activation elicited by the combination of p300 and/or GRIP-1 with HNF4 α 1 or HNF4 α 7. Numbers in parentheses show the fold factor induction upon addition of p300 and/or GRIP-1 over that of HNF4 α 1 or HNF4 α 7 alone. Repression induced by SMRT addition (see below) is expressed in percentage relative to HNF4 α alone. B: GRIP-1 can compete with SMRT in a promoter-specific fashion. 293T cells were transfected with the ApoB, the ApoCIII or the ApoAI reporter, 50 ng of the empty (pCB6), HNF4 α 1 or HNF4 α 7 expression vectors, 250 ng of pSG5.SMRT and/or 0, 50, 100, 200 or 300 ng of pSG5.GRIP-1 as indicated. Luciferase activity was normalized to β -galactosidase activity. All the experiments are shown as the mean \pm S.E.M. of a representative experiment performed in triplicate (some of the error bars are too small to be visible in the figure).

CAGCAAAGTTCAG and 5'GCTGAGGACGAAGAGATGGA, and for GRIP-1 5'ACACTAGCACCATGAGAGCC and 5'TCGC-TTGTCCAGTCAGATCC. Amplification was carried out for 25 and 18 cycles for SMRT/GRIP-1 and ribosomal RNA, respectively. 28S amplification was visualized with ethidium bromide. It was verified that the number of cycles chosen for PCR ensured analysis within the exponential phase of amplification for all of the primers used.

3. Results and discussion

Here, we have examined the effects elicited by coregulators upon transactivation by the HNF4 α isoforms expressed mainly in fetal (HNF4 α 7) and adult (HNF4 α 1) liver. Transfection experiments using reporters for HNF4 and expression vectors for the two isoforms coupled with GRIP-1, p300 and SMRT were performed. Since HNF4 α plays a major role in the regulation of Apo gene expression [19–21], three different constructs derived from the regulatory sequences of the ApoB, the ApoCIII and the ApoAI genes were used.

3.1. Robust ApoB transactivation by HNF4 α isoforms was largely independent of interacting partners

Fig. 1 presents the patterns of response of each reporter in cotransfection assays in concert with coactivators of the p300 and the p160 (GRIP-1) family of coactivators as well as with the SMRT corepressor. ApoB was very strongly activated by HNF4 α 1, and less strongly by HNF4 α 7. In the presence of either HNF4 α isoform, there was a weak but significant enhancement by p300 (two- to three-fold) and no effect of GRIP-1. In the case of SMRT, only HNF4 α 1 was subject to repression and the effect was of 25%. Neither of the assays

we devised to test for coactivation by GRIP-1 was positive with the ApoB reporter: only additive effects were observed when p300 and GRIP-1 were tested together, and GRIP-1 was unable to titrate SMRT-mediated repression (Fig. 1). These results are in line with the observation that GRIP-1 competes with SMRT through binding only of HNF4 α [5] and not SMRT itself. In addition, they provide a confirmation that GRIP-1 and, in the case of HNF4 α 7, SMRT as well are inactive in HNF4 α -mediated transactivation/transrepression of the ApoB promoter.

3.2. The ApoCIII reporter was moderately transactivated by both HNF4 α isoforms and by p300 while GRIP-1 and SMRT effects were weak but significant

In contrast to ApoB, transactivation of ApoCIII by HNF4 α 1 and HNF4 α 7 was only moderate (Fig. 1A). In addition, p300 provoked a large increase over the activity obtained with HNF4 α alone. However, the GRIP-1 effect was very small, and the two factors together resulted in purely additive effects (Fig. 1A). Concerning SMRT, the effects obtained were different with HNF4 α 1 and HNF4 α 7. In the presence of the HNF4 α 1 isoform, SMRT caused a significant inhibition that was effectively titrated by the addition of increasing amounts of GRIP-1, even though GRIP-1 itself had only weak transactivation capacity (Fig. 1B). In the presence of the fetal HNF4 α 7 isoform, SMRT had less of an effect and the addition of increasing amounts of GRIP-1 was unable to compete the SMRT repression, even though a positive effect of GRIP-1 alone was manifest (Fig. 1B).

3.3. The ApoAI reporter was strongly transactivated by HNF4 α isoforms and by p300 and GRIP-1, while the significant repression by SMRT was totally competed by GRIP-1

The ApoAI reporter was the most responsive to both HNF4 α isoforms and the coregulators tested (Fig. 1). Both isoforms strongly activated this reporter, although as observed for each of the promoters used here, HNF4 α 1 was more effective than HNF4 α 7. Significant activation was mediated by p300 and GRIP-1, and the combination of the two factors induced a synergistic response, but only in combination with HNF4 α 1. Indeed, only HNF4 α 1 contains two functional activation domains, AF1 and AF2, which could be a prerequisite for such synergy [7,22]. SMRT repression was significant with both isoforms, and in both cases a dose-dependent competition of repression was obtained with GRIP-1.

3.4. Transcript levels of the SMRT and GRIP-1 coregulators varied during liver development

The activity of transcription factors may be controlled in part by the physiological levels of expression of coregulators. Transcript levels of SMRT, nuclear receptor corepressor (NCoR) and other coactivators can vary from one cell type to another and during differentiation [23,24]. Therefore, we analyzed the levels of transcripts encoding SMRT and GRIP-1 in the liver.

Fig. 2 shows the timing of expression of HNF4 α isoforms (see [4]) and the results of semi-quantitative RT-PCR for SMRT and GRIP-1 transcripts carried out on total RNA from mouse liver of 14.5 days post-coitum (d.p.c.), newborn, 2 weeks old and adult. It was surprising to observe that SMRT and GRIP-1 transcripts were respectively undetectable

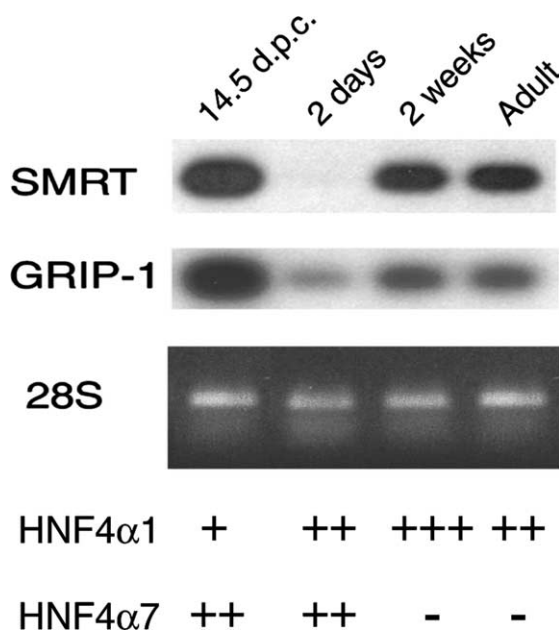


Fig. 2. SMRT transcripts are down-regulated in the liver at birth. RT-PCR analysis of total mouse liver RNA from 14.5 d.p.c., 2 days and 2 weeks after birth, and adult. The same cDNA samples were used to amplify SMRT, GRIP-1 and ribosomal 28S RNA as internal control. RT-PCR was performed with two independent liver RNAs and the results obtained were the same. SMRT transcripts at day 2 remained undetectable even after overexposure of the autoradiogram (data not shown). Below are shown the relative accumulations of transcripts of the two isoforms of HNF4 α determined using the same RNA samples (experiment originally published as figure 5 in [4] and summarized here).

or down-regulated just after birth. It should be pointed out that at 14.5 d.p.c., when SMRT and GRIP-1 transcripts were maximal, the liver is heavily populated by hematopoietic cells, which could be expressing these transcripts. This raises the possibility that expression of the coactivators in hepatocytes occurs only after birth, when the liver is no longer a hematopoietic organ. Even after overexposure of the autoradiogram, SMRT transcripts were undetectable just after birth, a time when the liver is undergoing profound changes in gene expression pattern, and when the activation of adult liver functions has just taken place. Indeed, for many of these functions HNF4 α is known to play a decisive role [25] in maintenance of expression. In the absence of the SMRT corepressor HNF4 α would be expected to have a more robust activating influence. GRIP-1 transcripts were down-regulated at birth as well; nevertheless, they were still present and, since p300 is more active than GRIP-1 in the context of coactivation potential in concert with HNF4 α (Fig. 1), a reduction in GRIP-1 concentration would not be expected to compromise HNF4 α -mediated activation of gene expression at birth.

3.5. Conclusions

The observations presented here reveal novel levels of control of gene expression in the liver mediated by HNF4 α , a gene product that has been demonstrated to be essential for expression of the differentiation program of the liver in the embryo and in the adult [20,26].

In order to understand the mechanism for the differences observed in the transactivation properties of the HNF4 α 1 and HNF4 α 7 isoforms, we have completed our earlier studies of *in vitro* interactions of HNF4 α proteins with coregulators [7] by cotransfection studies using the same interaction partners. In addition, by examining several different promoter constructs in parallel, with the initial aim of extending our previous observations, we observed promoter-specific differences in coactivation and corepression of target promoters that are as great as the differences exhibited by the two isoforms. While results from experiments that rely upon overexpression of transcription factors and their partners must be interpreted with caution, at present the *in vitro* and *ex vivo* approaches remain the only option when interactions among three molecules are being studied.

For this work we have relied upon three reporter constructs: two of them contain real promoter fragments of 400 (ApoB) and 850 (ApoCIII) bp, and one a multimerized HNF4 binding site from the ApoAI promoter. All of these regulatory sequences derive from genes that have been validated *in vivo* as genuine HNF4 α targets. Liver-specific deletion of all isoforms of HNF4 α in the mouse revealed that in 8.5 or 12.5 d.p.c. liver all three of the apolipoproteins are dramatically down-regulated upon ablation of the HNF4 α gene [19,26]. Specific deletion of HNF4 α in adult liver results in complete loss of ApoCIII expression [20]. Furthermore, the levels of ApoB and ApoCIII transcripts increase during development to reach the levels characteristic of adult liver [27]. Thus, the transcriptional responses that we have obtained using promoter studies recapitulate those observed during development: expression is lower during embryonic/fetal life when the less responsive HNF4 α 7 isoform is expressed.

Our results show that GRIP-1 activity on HNF4 α is promoter-specific: no activity with ApoB, only slight activity for ApoCIII and a stronger effect on ApoAI was observed. More-

over, one case of synergy through the action of the p160 and p300 family of coactivators was observed but appeared to exhibit the following requirements: (a) the activity of p300, (b) substantial activity of GRIP-1 and (c) both AF modules. These observations suggest that p300 acts as a more general coregulator of transcription, while p160 coactivators may be important as promoter-specific determinants of nuclear receptor activity. These results are in line with what has been previously suggested for the p160 coactivators [28].

Relevant transcriptional differences between HNF4 α 1 and HNF4 α 7 have been documented [7] and could account for the promoter-specific effects that we now report. Namely, HNF4 α 1 exhibits enhanced protease resistance compared to HNF4 α 7, but only upon specific DNA binding, suggesting that HNF4 α 1 folds differently when it binds to DNA. Indeed, such a change could confer a different affinity for corepressors and/or coactivators in the context of particular target promoters. Moreover, HNF4 α 7 possesses only one activation function, AF2, that can interact with both coactivators and corepressors. In contrast, HNF4 α 1 possesses two activation functions, AF1 being able to interact only with coactivators and AF2 with both classes of coregulators (see table 1 in [7]).

The HNF4 α 1 isoform is more strongly affected in its transactivation properties by coregulators than the embryonic HNF4 α 7 isoform, in accord with the fact that the promoters of genes encoding metabolic functions of the adult liver are often inducible. Moreover, only HNF4 α 1 possesses a functional AF1 that could act to antagonize SMRT repression [7]. Transcript levels of coregulators such as SMRT and GRIP-1 that act negatively or weakly on the promoters examined are reduced at birth (Fig. 2), the time when ApoCIII and many other genes in the liver are strongly activated. Thus, the effects of coregulators on HNF4 α activity *in vivo* could be determined by their levels of expression. Alternatively, discrimination between activators or repressors within a single cell could be modulated by the context of the target promoter and/or by differences in the affinity displayed by isoforms of the same nuclear receptor versus these coregulators.

In summary, we show here for the first time in a comparative study that the effects mediated by HNF4 α isoforms in concert with three different coregulators result in promoter-specific responses, adding a novel level of sophistication to the regulation of liver-specific gene expression. Indeed, the potential physiological relevance of the interaction of HNF4 α with these coregulators is highlighted by the fact that target genes of the same transcription factor are activated/repressed in a different fashion according to the combination of coregulators recruited to target promoters.

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